

Intracellular and *in Vitro*-Translated 27-kDa Proteins Contain the 3C-like Proteinase

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The coronavirus mouse hepatitis virus-A59 (MHV-A59) encodes a serine-like proteinase (3C-like proteinase or 3CLpro) in ORF 1a of gene 1 between nucleotides 10209 and 11114. We previously have demonstrated that proteins expressed *in vitro* from a cDNA clone of the 3CLpro region possess proteinase activity, and that the proteinase is able to cleave substrate *in trans*. We sought to determine if the 27-kDa *in vitro* cleavage product (p27) was an active form of the 3CLpro and whether this was consistent with the 3CLpro expressed in virus-infected cells. Antibodies directed against the 3CLpro domain detected 27-kDa MHV proteins *in vitro* and in MHV-A59-infected cells. The 27-kDa proteins were able to cleave substrate *in trans* without other protein cofactors or supplemental membranes, and the p27 proteinase activity was retained after purification by immunoprecipitation and gel electrophoresis. When p27 was expressed *in vitro* with portions of the amino- and carboxy-terminal flanking domains (MP1 and MP2), p27 was not liberated by *cis* cleavage. The proteolytic activity of the 27-kDa proteins was inhibited by a variety of cysteine and serine proteinase inhibitors, and was eliminated by the cysteine proteinase inhibitor E64d. These results indicate that the 27-kDa protein is a mature proteinase in MHV-A59-infected cells, and that appropriate processing of this molecule occurs *in vitro*. © 1996 Academic Press, Inc.

INTRODUCTION

The coronavirus mouse hepatitis virus-A59 (MHV-A59) contains a 32-kb single-stranded RNA genome of positive polarity. Intracellular replication of MHV is initiated by translation of gene 1 of the genome RNA into a polyprotein with an estimated mass of greater than 750 kDa. The gene 1 polyprotein is a fusion protein that is assumed to be the result of translation through two out-of-frame open reading frames, ORF 1a and ORF 1b, by a ribosomal frameshift (Pachuk *et al.*, 1989; Lee *et al.*, 1991). The gene 1 polyprotein is thought to incorporate all the activities necessary for MHV RNA replication, including three proteinases that are predicted to mediate all the maturational cleavages of the polyprotein (Lee *et al.*, 1991; Gorbalenya *et al.*, 1991; Gorbalenya and Koonin, 1993). It is known that proteolytic processing of the gene 1 polyprotein is required for MHV replication, and that inhibition of proteolytic activity also results in rapid shutoff of MHV RNA synthesis (Kim *et al.*, 1995).

Three proteinases have been predicted to be encoded within the MHV gene 1; two of the proteinases have been experimentally confirmed by *in vitro* expression and activity. A papain-like proteinase, encoded between nucleotides 3559 and 4049 from the 5' end of gene 1, is known to cleave at the carboxy terminus

of p28 (Baker *et al.*, 1993; Dong and Baker, 1994; Hughes *et al.*, 1995). The papain-like proteinase has not been identified in MHV-infected cells; however, based on immune precipitation of MHV proteins from infected cells using specific antisera, it is likely that this activity is contained in either the 50- or the 240-kDa MHV gene 1 proteins (Denison *et al.*, 1992; Bonilla *et al.*, 1995).

We have identified a second MHV proteinase that is encoded between nucleotides 10209 and 11114 from the 5' end of gene 1 (Fig. 1). *In vitro* translation of a cDNA clone encoding the predicted MHV-A59 3C-like proteinase (3CLpro) and portions of the flanking domains (MP1 and MP2) results in expression of a proteinase and liberates a 27-kDa protein (p27) (Lu *et al.*, 1995). p27 is cleaved *in trans* at an amino-terminal glutamine-serine (QS₃₃₃₃₋₄) dipeptide. Although mutagenesis of predicted catalytic cysteine₃₄₇₈ or histidine₃₃₇₄ residues within the 3CLpro domain abolished proteolytic activity of proteins expressed *in vitro*, it was not known if the 27-kDa protein was the active proteinase.

In this study we sought to determine if the active 3CLpro *in vitro* and in virus-infected cells was the 27-kDa protein previously identified *in vitro*. Antisera specific to the 3CLpro domain detected 27-kDa (p27) proteins both *in vitro* and in cells. Gel-purified p27 proteins from cells or *in vitro* translation reactions were able to cleave substrate *in trans*, whereas the *in vitro*-expressed 3CLpro was not able to liberate p27 *in cis*.

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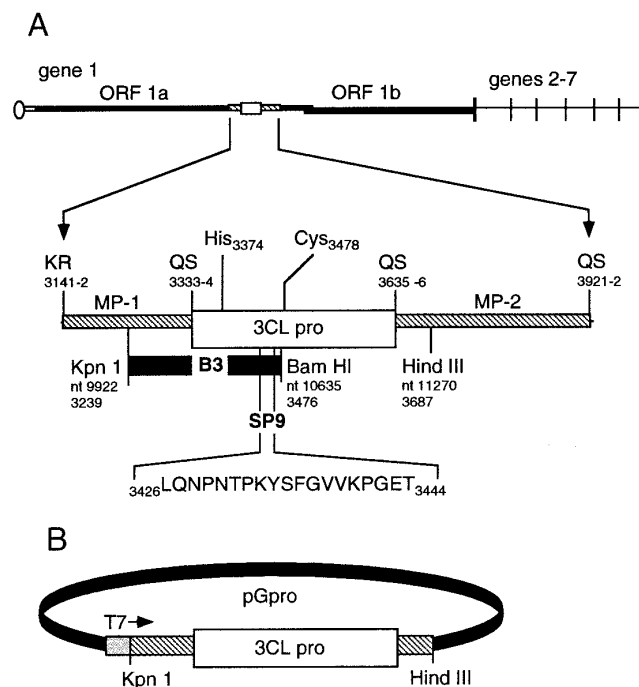


FIG. 1. Location of putative MHV-A59 3CLpro domain and 3CLpro antibodies. (A) The MP-1/3CLpro/MP-2 coding region is shown both by nucleotide (nt) number from the 5' end of the genome, as well as by amino acid residue number from the amino terminus of the gene 1 polyprotein, after the sequence of Bonilla *et al.* (1994). Numbers represent amino acid residue number unless preceded by "nt." The 3CLpro-encoding construct pGpro encodes residues 3239–3687. The B3 anti-serum was induced against the fusion protein spanning residues 3239–3476. The SP9 antiserum was raised against a synthetic peptide as shown. Locations of essential His and Cys residues are shown, as well as the confirmed amino-terminal QS cleavage site of 3CLpro and the predicted carboxy-terminal QS dipeptide. (B) pGpro, the parent construct for *in vitro* translation of the 3CLpro, incorporates the *KpnI*–*HindIII* fragment behind a T7 promoter in pGEM 3Zf⁺.

The proteolytic activity of p27 was inhibited by proteinase inhibitors, with complete inhibition occurring with the cysteine proteinase inhibitor E64d. We were therefore able to confirm that the p27 protein detected in virus-infected cells and *in vitro* was an active form of the 3C-like proteinase.

MATERIALS AND METHODS

Infection of DBT cells with MHV-A59

DBT cells were infected with MHV-A59 at a multiplicity of infection (m.o.i.) of 20 PFU/cell in DMEM 2% FCS (Denison *et al.*, 1992). Methionine-free DMEM (Gibco) containing 2% FCS and 10 μ g/ml actinomycin D (Sigma) were added to the cells at 3 hr postinfection (p.i.). Intracellular proteins were labeled from 6 to 8 hr p.i. with 200 μ Ci/ml [³⁵S]methionine (Expre³⁵S³⁵S protein labeling mix; DuPont, NEN), unless otherwise indicated. During experiments to determine the time of appearance of p27, proteins were labeled for 2-hr intervals between 2 and 12 hr

p.i. During proteinase-inhibition experiments, proteinase inhibitors were added to the media 1 hr before the addition of radiolabel. During pulse–label and pulse–chase experiments, synchronization of translation was achieved by the addition of NaCl (200 mM) to the media for 30 min at 5.5 hr p.i. (Denison *et al.*, 1992). The high-salt medium was replaced with isotonic medium containing [³⁵S]Met, and polypeptides were labeled for various times from 15 to 120 min. The infected monolayers were then harvested or alternatively chased with excess cold methionine for an additional 90 min.

Preparation of antigens and antibodies.

We raised antisera directed against the MHV 3CLpro by immunizing rabbits with a keyhole limpet hemocyanin (KLH)-linked oligopeptide or a fusion protein within the 3CLpro region of gene 1 (Fig. 1). A synthetic 19-amino-acid oligopeptide (3426-LQNPNTPKYSFGVVKPGET-3444) was dissolved in phosphate-buffered saline and coupled to KLH (Sigma) using glutaraldehyde. The fusion protein spanned residues 3239 to 3476 of the ORF 1a polyprotein, which included 95 amino acids upstream of the 3CLpro amino-terminal serine and extended to within 2 amino acids of the essential cysteine₃₄₇₈. The plasmid pGEM-K/B was constructed by subcloning a 713-nucleotide *KpnI*–*BamHI* restriction fragment of ORF 1a from nt 9922 to 10635 into pGEM 3Zf⁺. A 735-nucleotide *KpnI*–*PstI* restriction fragment was isolated from pGEM, ligated to *KpnI*–*PstI*-digested pQE-30 plasmid (Qiagen) and used to transform *Escherichia coli* M15(pREP4) cells. Bacterial expression and purification of the recombinant protein were performed according to the manufacturer's instructions. Antisera directed against the oligopeptide and fusion protein were obtained from Cocalico. The antisera were named Sp9 (oligopeptide) and B3 (fusion protein).

Immunoprecipitation and electrophoresis

Immunoprecipitation (IP) of MHV proteins was performed as previously described (Denison *et al.*, 1992). Briefly, whole-cell lysates of MHV-A59-infected DBT cells (equivalent to 10⁶ cells) or *in vitro* translation products (5 \times 10⁶ TCA-precipitable counts) were diluted into 1 ml of radioimmunoprecipitation assay (RIPA) buffer to obtain a final concentration of SDS of 0.1%. Polyclonal antisera, 4 μ l of PMSF (phenylmethylsulfonyl fluoride; Sigma) (20 mg/ml), and 30 μ l of preswollen protein A–Sepharose beads (Sigma) were added directly to the reaction mix. After rocking for 2 hr at 4°, the supernatant was removed and protein A bead/antibody/antigen complexes were washed four times with alternating high (1 M NaCl) and low (100 mM NaCl) salt RIPA buffer. The beads were boiled in 50 μ l of 2 \times Laemmli buffer for 5 min before electrophoresis on a 5–18% gradient SDS–polyacryl-

amide gel (Laemmli, 1970). Radiolabeled proteins were visualized by fluorography.

In vitro transcription and translation

Recombinant plasmids were transcribed and translated *in vitro*, as previously described (Lu *et al.*, 1995). Standard reactions were performed in a total volume of 25 μ l, with 0.5 μ g of plasmid DNA and 20 μ Ci of [35 S]Met (Amersham) for 90 min at 30°. Reactions were terminated by addition of 2 \times Laemmli buffer or quick freezing in dry ice/ethanol and storage at -70°.

In vitro trans cleavage assay

DBT cells were infected with MHV-A59 at an m.o.i. of 20 PFU/cell. At 6 to 8 hr postinfection, nonradiolabeled whole-cell lysates (4×10^6 cells) were immunoprecipitated with antiserum Sp9 or preimmune serum from the same rabbit. The pGpro construct, which expresses an active proteinase, was translated *in vitro* without radiolabel for 1 hr, and translation products were immunoprecipitated with antiserum Sp9. Immunoprecipitation products were separated on a 5–18% gradient SDS–polyacrylamide gel that was prerun with 0.1 mM thioglycolate in the upper running buffer (100 V, 15 min). The 27- and 32-kDa bands from pGpro translation were located by comparison with prestained protein markers and radiolabeled pGpro translation products, and were excised from the gel. The 27-kDa bands from IP of infected cell lysates using Sp9 or preimmune serum were also excised. The gel slices were crushed in microcentrifuge tubes using pipette tips. A site-directed mutant of pGpro that lacks proteinase activity but contains cleavage sites for the 3CLpro (pGproG41) (Lu *et al.*, 1995) was translated in the presence of [35 S]Met for 1 hr, and a portion of the translation lysate was mixed directly with each gel slice and incubated for 4 hr at 30°. Samples from the incubated lysate were analyzed by electrophoresis in 5–18% SDS gradient gels and visualized by fluorography. During experiments to determine if the proteolytic activity could be inhibited, proteinase inhibitors such as 2 mM PMSF, 100 μ g/ml E64c (Sigma), or 1 mM ZnCl₂ (zinc chloride; Sigma) were added into the mixtures containing radiolabeled pGproG41 and cold 27-kDa protein.

Cis cleavage assay

pGpro was expressed in the presence of [35 S]Met for 40 min in the combined transcription/translation reaction. The translation lysates were then mixed with dilution buffer (50 mM Tris–acetate, pH 7.5, 5 mM magnesium acetate, 5 mM dithiothreitol, 0.2 mM disodium ethylenediamine tetraacetate, and 100 mM potassium acetate) in serial dilutions from 1:2 to 1:320, and incubated for an

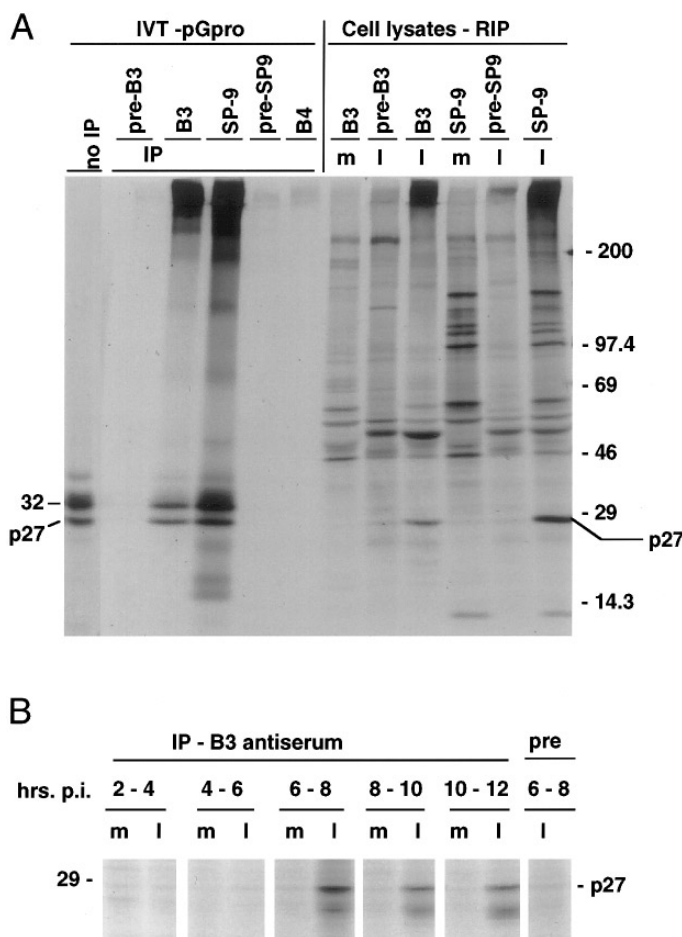


FIG. 2. Detection of A59 3CL proteinase *in vitro* and in virus-infected cells. (A) Infected and uninfected cell lysates and *in vitro* transcription–translation products of pGpro were immunoprecipitated with the antisera as indicated. IP products were analyzed by 5–18% gradient SDS–PAGE. Samples of the IVT were directly analyzed without IP in the “no IP” lane. Pre, preimmune serum from the rabbit used to raise specific antisera; B4, an unrelated gene 1 antibody; M, mock-infected cells; and I, infected cell lysates. Molecular mass markers and specific precipitated polypeptides are indicated to the right. (B) Radiolabeled methionine was added to the medium for 2 hr at the times p.i. indicated above the lanes. Lysates of infected cells (I) and mock-infected cells (m) were immunoprecipitated with antiserum B3 or preimmune serum (pre). Samples were analyzed by 5–18% gradient SDS–polyacrylamide gels. Molecular mass markers are to the left and the size of the specific precipitation product is to the right.

additional 120 min at 30°. Diluted lysates were concentrated using a Centricon-10 concentrator (Amicon, Inc.). Concentrated samples were electrophoresed on 5–18% gradient SDS–polyacrylamide gels, the gel was treated with DMSO/PPO, and the proteins were visualized by fluorography.

RESULTS

Identification of 27-kDa proteins *in vitro* and in MHV-A59-infected cells

Antisera Sp9 and B3 were used to immunoprecipitate MHV-A59 gene 1 proteins from *in vitro* translation prod-

ucts of pGpro and from MHV-infected cells. Preimmune sera, uninfected cell lysates, and antibodies directed against other regions of the gene 1 polyprotein were used as controls for specificity. Major processed polypeptides of 32 and 27 kDa were expressed from pGpro *in vitro*, as previously described (Lu *et al.*, 1995). Both the 32- and the 27-kDa proteins were detected by B3 and Sp9 antisera (Fig. 2). Both antisera also precipitated products of greater than 200 kDa that were not detected by preimmune sera. Since the maximum possible size of the expression construct was 48 kDa, these bands probably represented aggregates. No proteins were detected by the unrelated B4 antiserum. Thus it appeared that both B3 and Sp9 were specific for the 3CLpro region and were able to detect p27.

From infected cell lysates, both antisera precipitated a virus-specific protein that had the same apparent mass (27 kDa) as the *in vitro* cleavage product. The intracellular p27 was not detected in mock-infected cell lysates, nor was it precipitated from infected cells by preimmune serum. Multiple larger polypeptides were precipitated by both B3 and Sp9; however, p27 was the only protein uniquely detected by immune sera in infected cells. The results demonstrated that p27 was a specific product of the 3CLpro domain both *in vitro* and in virus-infected cells.

We next sought to determine the kinetics of p27 synthesis in virus-infected cells. Our studies of other MHV gene 1 products have shown that they are first detected at 4 to 6 hr postinfection and are present in larger amounts as the infection progresses, suggestive of accumulation or increasing rates of synthesis due to amplification of genome late in infection. The B3 antiserum was used to immunoprecipitate whole-cell lysates of infected and mock-infected cells labeled with [³⁵S]Met at different times p.i. p27 was first detected at 6–8 hr postinfection, (Fig. 2). This pattern was consistent with the earliest appearance of all other identified MHV gene 1 proteins (Denison *et al.*, 1992). The p27 protein from lysates of 1×10^5 cells was easily detected by fluorography of SDS gels within 12 hr. An additional faint band of approximately 27 kDa was visible during every labeling period from 0 to 12 hr, from B3-precipitated, mock-infected cell lysates and in infected cells precipitated by preimmune serum, suggesting that it was probably a cell protein that comigrated with the MHV p27.

p27 processing and stability in MHV-infected cells

We next sought to determine whether p27-containing precursors could be identified, and to assess the stability of p27 following cleavage. Intracellular gene 1 translation was synchronized by pretreatment of cells with hypertonic NaCl, and proteins were radiolabeled for intervals from 15 to 120 min (Fig. 3). At each time point one plate was harvested (pulse-label), and another was chased

with excess unlabeled methionine for an additional 90 min (variable pulse/constant chase). Infected and mock-infected cell lysates were immunoprecipitated with anti-serum Sp9. A third set of infected cell monolayers was radiolabeled for 60 min, and translation was terminated with cycloheximide followed by additional incubation for up to 3 hr (pulse-chase).

The variable pulse/constant chase experiment (Fig. 3B) demonstrated that radiolabel was incorporated into p27 between 60 and 75 min after initiation of translation. The pulse-label (A) showed that p27 was cleaved between 60 and 75 min after addition of radiolabel. Densitometric analysis of the bands showed no significant difference between the amount of p27 detected in the pulse-label or pulse-chase translations at 60 and 75 min (data not shown), indicating that the time of incorporation of radiolabel into the polypeptide and the time of cleavage were closely spaced. Thus p27 appeared to be cleaved soon after synthesis in infected cells. In contrast, the cleavage of p27 during *in vitro* translation of the pGpro construct has been shown to be delayed for 30 to 60 min after incorporation of radiolabel (Lu *et al.*, 1995). The reason for the difference in the rates of cleavage *in vitro* and in cells is not known.

In the pulse-chase experiment (Fig. 3C), after p27 was cleaved, the amount detected by immunoprecipitation remained constant for at least 2 hr. Since it appeared that p27 was rapidly processed after synthesis, this suggested that p27 was relatively resistant to degradation in infected cells.

We could not identify precursors to p27 in any of these experiments. It is possible that p27 was rapidly cleaved from the growing polyprotein with no intermediate precursors. However, both Sp9 and B3 precipitated a large number of other proteins that may have obscured p27-containing precursors. We are currently using the Sp9 peptide to obtain mAbs to the 3CLpro domain, that can be used to further investigate possible p27 precursors.

Inhibition of p27 processing in cells

We next investigated whether p27 processing in cells could be blocked by proteinase inhibitors. Infected DBT cells were radiolabeled in the presence of leupeptin, PMSF, E64d, *N*-ethylmaleimide (NEM), or ZnCl₂, and cell lysates were immunoprecipitated with Sp9 antiserum. Both serine and cysteine proteinase inhibitors affected proteolytic processing of p27 in DBT cells during MHV infection (Fig. 4). Leupeptin, PMSF, and NEM all consistently inhibited p27 processing between 50 and 85%, whereas E64d inhibited processing 100%. E64d has been previously shown to inhibit MHV RNA synthesis and virus replication in DBT cells at the same concentration used in this experiment (Kim *et al.*, 1995). There was no detectable inhibition of p27 processing in the presence of 0.1 mM ZnCl₂. This concentration was the highest that could be used without cellular cytotoxicity,

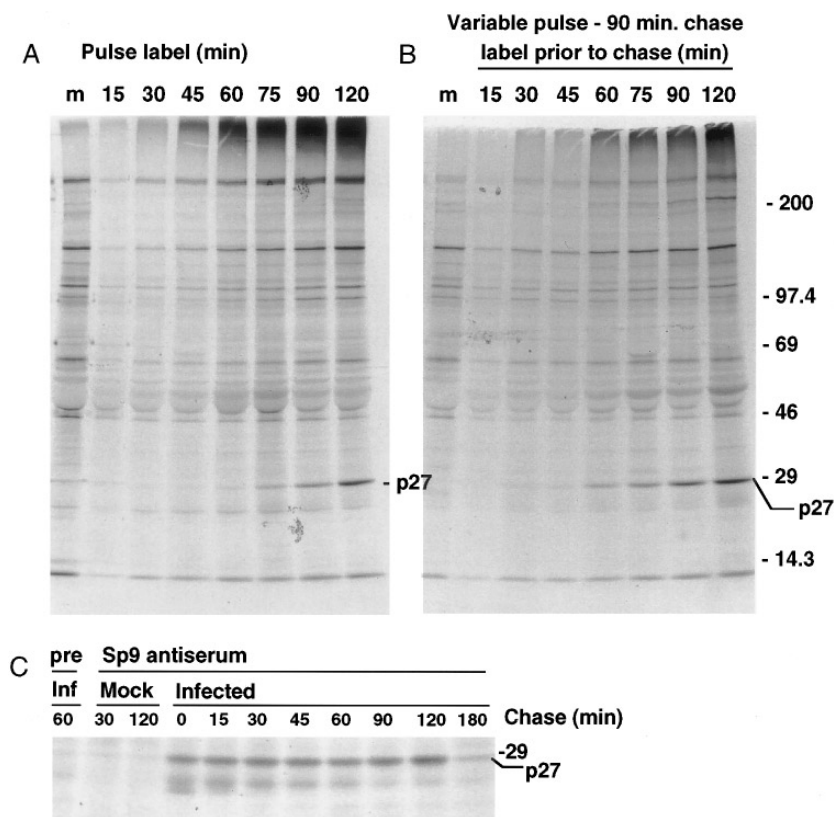


FIG. 3. Synthesis, processing, and stability of p27 in cells. At 5.5 hr postinfection, infected cells were incubated in medium containing 200 mM NaCl for 30 min. The hypertonic medium was replaced with isotonic medium containing [35 S]Met for the times in minutes indicated above the lanes. (A) Pulse-label: cells were harvested at the end of the labeling period in minutes as indicated. (B) Variable pulse/constant chase: at the end of the labeling period, reactions were chased with excess unlabeled methionine for an additional 90 min. As a control, replicate plates of mock-infected cells (m) were labeled for 60 min with or without the 90-min chase. Whole-cell lysates were immunoprecipitated with antiserum Sp9. Molecular mass markers are shown to the right and the location of p27 is indicated next to each gel. (C) Pulse-chase: following synchronization, proteins were radiolabeled for 60 min. Incorporation was terminated by addition of cycloheximide and excess cold methionine, and samples were chased for the time in minutes shown above the gel. Proteins were immunoprecipitated with Sp9 antiserum and separated on a 5–18% SDS gel. The location of p27 and the location of marker proteins are shown. Inf, infected cells; pre, Sp9 preimmune rabbit serum; Mock, mock-infected cell lysates.

but was much lower than the concentrations used (1–2 mM) to inhibit MHV gene 1 proteinases *in vitro* (Denison *et al.*, 1992). No p27 precursors were detected in the presence of these inhibitors.

Trans proteolytic activity of p27 from IVT and infected cells

The MHV 3CLpro has been predicted to include amino acids from S₃₃₃₄ to Q₃₆₃₅, with an estimated mass of 34 kDa (Lee *et al.*, 1991). In contrast, we have consistently detected proteins with an estimated mass of 27 kDa, both from infected cells and from *in vitro* translation (IVT) products of pGpro; we have also shown that the amino terminus of *in vitro*-translated p27 is the QS dipeptide predicted to be the cleavage site of the 3CLpro. Therefore, we sought to determine whether the 27-kDa proteins we identified in cells and *in vitro* were an active form of the 3CLpro.

We used an *in vitro trans* cleavage assay to assess p27

proteolytic activity. Nonradiolabeled p27 proteins from *in vitro* translation of the construct expressing active proteinase (pGpro) and from virus-infected cells were excised from the SDS-PAGE gels and incubated with IVT lysates containing radiolabeled proteins translated from the pGproG41 mutant (His41-Gly). pGproG41 lacks proteinase activity but is still a cleavage substrate for the *trans* active 3CLpro *in vitro*, resulting in the appearance of new p27 molecules (Lu *et al.*, 1995). The pGproG41 translation reactions were terminated by the addition of RNase, cycloheximide, and excess cold methionine before incubation with the p27-containing gel slices. As controls, pGproG41 translation lysates were incubated with gel fragments lacking protein bands, as well as with gel slices containing 27-kDa region proteins precipitated from MHV-infected cells by preimmune sera.

The results obtained during *in vitro* translation of pGpro and pGproG41 were consistent with our previous study (Fig. 5) (Lu *et al.*, 1995): pGpro translation resulted in processing of a prominent 27-kDa band that was ab-

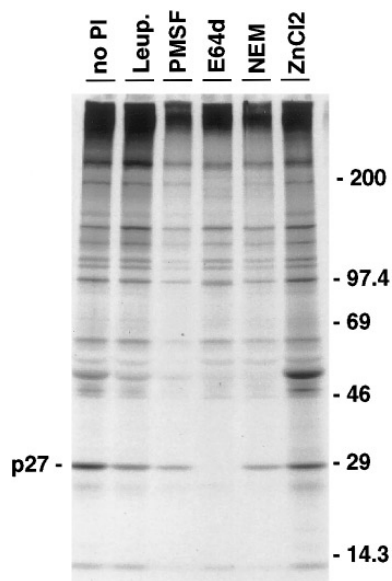


FIG. 4. Inhibition of p27 proteinase activity. Infected DBT cells were incubated with different proteinase inhibitors (4 mM leupeptin, 2 mM PMSF, 400 μ g/ml E64d, or 0.1 mM zinc chloride) for 1 hr before and during labeling with [35 S]Met, from 6 to 8 hr postinfection. Whole-cell lysates of infected cells were immunoprecipitated with the oligopeptide antiserum Sp9. Infected cells without proteinase inhibitor (no PI) were used as a control. Molecular mass markers are to the right and p27 is indicated the left.

sent from the translation reaction containing the pGproG41 mutant construct alone (lane 1). However, when the pGproG41 translation products were incubated with the gel-purified MHV p27 proteins immunoprecipitated from cells or from IVT lysates containing pGpro translation products, new molecules of radiolabeled p27 were generated (lanes 3 and 4). Since only the pGproG41 translation products were labeled, the new p27 molecules were the result of *trans* cleavage by the nonlabeled p27 proteins in the gel slices. In contrast, when pGproG41 IVT products were incubated with gel fragments containing the 27-kDa region proteins obtained by immunoprecipitation of infected cells by preimmune serum, no cleavage of p27 was observed (lane 2). Together these results demonstrated that the proteolytic activity was specific to the 27-kDa protein precipitated by B3 and Sp9. The comigrating 27-kDa protein from mock-infected cells and from preimmune serum-precipitated infected cells did not possess proteinase activity. The 32-kDa translation product of pGpro IVT also failed to cleave new molecules of p27 (lane 5), even though this band was closer in size to the predicted mass of the MHV 3CLpro and appeared to be a specific proteolytic product *in vitro*. However, no protein of 32 kDa was identified in virus-infected cells, suggesting that this band may be an aberrant product of *in vitro* translation of pGpro. Only the virus-specific p27 proteins cleaved new molecules of p27.

When proteinase inhibitors were incubated with the

gel slices containing the p27 proteinase before addition of substrate, processing was decreased or abolished (lanes 6–11). PMSF completely inhibited p27 cleavage activity, compared with the partial inhibition seen in virus-infected cells. E64c completely inhibited cleavage activity of p27, similar to the result obtained in virus-infected cells. E64c is the active form of E64 to which E64d is converted on entry into cells. The lowest concentration of E64c used (100 μ g/ml or 300 μ M) was 25% of that required to inhibit cleavage of p27 in cells, indicating that the proteinase was more sensitive to inhibition by E64 than previously thought. Zinc chloride inhibited proteolytic activity of p27 isolated from virus-infected cells, but did not inhibit activity of p27 isolated from *in vitro* translation products of pGpro. The reason for this was not clear, but was compatible with the less consistent results of ZnCl₂ inhibition seen in virus-infected cells.

Lack of *cis* cleavage activity of *in vitro*-translated 3CLpro

Having demonstrated that the mature p27 molecule was able to liberate other p27 molecules *in trans*, we sought to determine whether p27 could also be cleaved *in cis*. The pGpro construct was used to express the active proteinase along with portions of the flanking hydrophobic domains, as previously described (Lu *et al.*, 1995). Following 40 min of translation *in vitro*, the translation lysates were diluted from 2- to 320-fold with dilution buffer and incubated an additional 2 hr (Fig. 6). Cleavage of p27 was observed only in the undiluted and 2-fold-diluted samples, demonstrating that *cis* cleavage did not occur in the *in vitro* system using a partial construct of gene 1. It is still possible that *cis* cleavage may be a mechanism of cleavage of the 3CLpro early in infection,

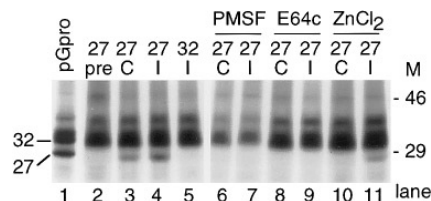


FIG. 5. *Trans* processing of substrate by intracellular and *in vitro*-translated p27. *In vitro* translation, intracellular protein labeling, IP, and *trans* cleavage assays are described under Methods and Materials. Lane 1 shows translation of pGpro alone, without IP. Lanes 2–11 show translation of the proteolytically inactive mutant pGproG41. The translation products were incubated with p27 within gel slices that were excised from SDS–polyacrylamide gels. The p27 proteins were nonradiolabeled translation products of intracellular MHV expression or *in vitro* translation of pGpro that had been immunoprecipitated with the 3CLpro-specific antiserum Sp9. Lanes 2–4 and 6–11 show results of the incubation of pGproG41 IVT products with 27-kDa proteins; I, p27 from IP of pGpro translation *in vitro*; C, p27 from IP of MHV-infected cell lysates. Lane 5 is an incubation with a 32-kDa protein from IP of pGpro IVT. Lanes 6–11 show the effect of addition of proteinase inhibitors to the *trans* cleavage assay: PMSF (2 mM), E64c (100 μ g/ml), ZnCl₂ (1 mM).

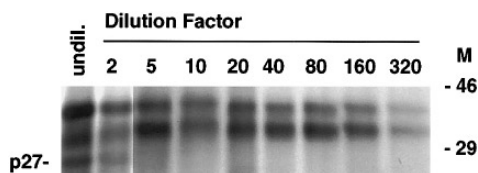


FIG. 6. Dilution analysis of p27 proteinase activity. pGpro was translated as described under Materials and Methods. Translation was halted at 40 min and lysates were diluted as shown above the figure. Undil., undiluted lysate; 320, 320-fold dilution. Samples were incubated an additional 120 min prior to electrophoresis. The location of p27 is shown to the left and the location of protein markers to the right.

but these results suggest that *trans* cleavage is the major mechanism by which the polyprotein is processed by the 3CLpro.

DISCUSSION

We have identified the active 3C-like proteinase molecules expressed from MHV-A59 ORF 1a *in vitro* and in virus-infected cells. The proteins have identical apparent masses of 27 kDa, smaller than either the predicted MHV 3CLpro (33 kDa) or the identified human coronavirus 229E 3CLpro (34 kDa). The MHV 3CLpro has been predicted to span amino acids from S₃₃₃₄ to Q₃₆₃₅, and to have a mass of 34 kDa (Gorbalenya *et al.*, 1989a,b; Lee *et al.*, 1991), similar to that predicted for the HCV 229E 3CLpro. A 34-kDa protein has been detected in 229E-infected cells, using antibodies specific to the 3CLpro region (Ziebuhr *et al.*, 1995). The 229E 34-kDa protein from infected cells has not been shown to possess proteinase activity; however, a 229E 34-kDa protein expressed in *E. coli* from a cDNA clone encoding the predicted 3CLpro was able to cleave substrate.

We have not determined the reason for the discrepancy between the observed and predicted masses of the MHV 3CLpro. Gel analysis may underestimate the molecular mass of proteins, especially if the protein is not completely denatured, or if the protein binds a disproportionately large amount of SDS with a concomitant increase in negative charge. We believe that the p27 proteins were completely denatured, since translation products were boiled extensively in SDS-containing loading buffer before electrophoresis. Further, boiling the samples in loading buffer containing 8 M urea, followed by electrophoresis on urea-containing gels, did not alter the apparent molecular mass of the 27-kDa proteins (data not shown). We are currently attempting to resolve this question by identifying the carboxy-terminal cleavage site of p27 and by assessing the size and proteolytic activity of truncated versions of the predicted full-length 3CLpro.

Despite the discrepancy in predicted and apparent mass, we have shown that the 27-kDa proteins from cells and *in vitro* translation lysates were identical in mobility, had the same pattern of antibody detection, and were

active proteinases. In addition, purification of the 27-kDa proteins by immunoprecipitation and SDS-polyacrylamide gel electrophoresis did not eliminate their ability to process other molecules of p27 *in trans*. Cellular proteinases involved in hemostasis have been shown to retain or regain activity after SDS gel electrophoresis (Wagner *et al.*, 1985; Tans *et al.*, 1989). However, there is a paucity of analogous studies with the viral proteinases. Proteinase activities from hepatitis C virus and yellow fever virus have been demonstrated in cell lysates. Amberg *et al.* reported *trans* cleavage activity of the yellow fever virus NS2B-3 in lysates of virus-infected BHK cells, using an *in vitro*-translated substrate (Amberg *et al.*, 1994). The *trans* cleavage activity of the hepatitis C virus NS3 serine proteinase has been demonstrated in lysates of HeLa cells. HCV NS3 was expressed in HeLa cells using a recombinant vaccinia virus system, and cell lysates were incubated with an *in vitro*-translated substrate (Bouffard *et al.*, 1995). However, the proteinase activity was not purified from the cell lysates in either of these studies. Our approach allowed us to demonstrate the proteolytic activity of a specific purified protein, something that has not previously been accomplished with a coronavirus proteinase.

The p27 protein precipitated from MHV-infected DBT cells was relatively abundant. We were able to detect p27 following immunoprecipitation of radiolabeled lysates from 1×10^5 cells by gel exposures of 12 hr. In contrast, the putative HCV 229E 3CLpro was reported to be present in very small amounts in infected cells, requiring prolonged exposures of 28 days to detect the protein (Ziebuhr *et al.*, 1995). It is not known whether these differences reflect the sensitivity of the antisera used or are true indicators of the amounts of the proteinases produced by the two viruses. MHV p27 was detected in cells beginning at 6 hr postinfection, similar to the other MHV gene 1 products identified in virus-infected cells. This also was distinct from the results obtained with the HCV-229E 3CLpro, which was detected at earlier time points, but declined at later times p.i. (Ziebuhr *et al.*, 1995).

Once processed, p27 was resistant to degradation for over 2 hr in infected cells and in reticulocyte lysates, suggesting that stability and accumulation of p27 may be important for processing of the gene 1 polyprotein. It was surprising that no p27-containing precursors were detected in MHV-infected cells. The B3 antiserum was raised against a fusion protein that extended into the domain flanking the amino terminus of p27, yet still did not detect precursors. Although it is possible that the available antisera were unable to bind to intermediate precursors to p27, or that background proteins obscured true precursors, the kinetics of labeling and processing support the conclusion that p27 was rapidly cleaved from the nascent polyprotein.

The proteolytic activity of p27 was most efficiently inhibited by derivatives of E64, an irreversible cysteine pro-

teinase inhibitor. E64 was developed as an inhibitor of cellular cysteine proteinases such as calpain, cathepsin L, and cysteine proteinases related to papain. Interestingly, it has not inhibited 3C proteinases of the picornaviruses such as 2A and 3C, whereas it has been used against papain-like proteinases such as the L proteinase of the picornavirus FMDV (Kleina and Grubman, 1992). The coronavirus 3CLpro domain has been called "3C-like" based on the orientation of histidine and cysteine residues, as well as predicted substrate binding pockets (Lee *et al.*, 1991). We have demonstrated the essential nature of the His and Cys residues; however, the coronavirus 3CLpro domain is predicted to have a much larger number of amino acid residues following the substrate binding pocket than the picornavirus 3C (Lu *et al.*, 1995). Although it has not been possible to classify cysteine proteinases based on their E64 sensitivity, our results suggest that the MHV p27 may have significant differences in structure or activity from the picornavirus 3C proteinases. Inhibition of p27 activity by E64 is also consistent with our study that demonstrated inhibition of MHV RNA synthesis and viral replication by E64d (Kim *et al.*, 1995). We do not know whether inhibition of liberation of p27 from the polyprotein is by itself enough to block replication; it is possible that E64 is blocking the ability of p27 to process polymerase molecules expressed from ORF 1b, leading to shutoff of viral RNA synthesis.

In conclusion, we have identified a 27-kDa MHV gene 1 protein both *in vitro* and in virus-infected cells that is the active 3CLpro. The proteinase is resistant to degradation in cells, and is able to act *in trans* to liberate additional proteinase molecules from the polyprotein. We believe these data and the methods used to generate them will allow us to more precisely determine the sites and mechanisms of action of this proteinase and to better characterize its role in coronavirus replication.

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